PARTIAL COMPLEMENTATION OF THE UV SENSITIVITY
OF _DEINOCOCCUS RADIODURANS_ EXCISION REPAIR MUTANTS
BY THE CLONED _denV_ GENE OF BACTERIOPHAGE T4

1990

YAO

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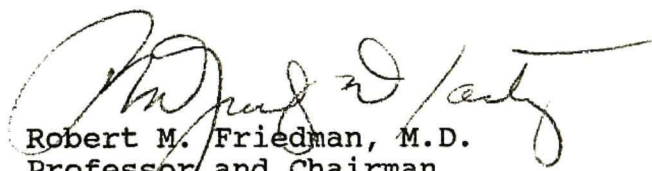BETHESDA, MARYLAND 20814-4799

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Title of Thesis: **"Partial complementation of the UV sensitivity of <u>Deinococcus radiodurans</u> excision repair mutants by the cloned <u>denV</u> gene of bacteriophage T4"**

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ABSTRACT

Title of Thesis: Partial complementation of the UV sensitivity of _Deinococcus radiodurans_ excision repair mutants by the cloned _denV_ gene of bacteriophage T4

Hanlong Yao, Master of Science, 1990

Thesis directed by: Kenneth W. Minton, Associate professor of Pathology

Deinococcus radiodurans has two endonucleases that incise UV irradiated DNA, UV endonuclease-α and UV endonuclease-β, that are believed to functionally overlap. Both endonucleases must be mutationally inactivated to yield an incisionless, markedly UV-sensitive phenotype. _denV_, the bacteriophage T4 gene encoding pyrimidine dimer-DNA glycosylase (PD-glycosylase), was introduced and expressed via duplication insertion in _D. radiodurans_ wild type, and single and double UV endonuclease mutants. Expression of _denV_ increased UV survival of an incisionless double mutant and the strain deficient only in UV endonuclease-β. The strain deficient in UV endonuclease-α has wild-type UV-resistance, and the expression of PD-glycosylase exerted no survival effect on this strain or wild-type. These results suggest that UV endonuclease-α does not recognize one or more types of cyclobutane dimer incised by the PD-glycosylase or UV endonuclease-β.

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PARTIAL COMPLEMENTATION OF THE UV SENSITIVITY OF

DEINOCOCCUS RADIODURANS EXCISION REPAIR MUTANTS

BY THE CLONED DENV GENE OF BACTERIOPHAGE T4

by

Hanlong Yao

Thesis submitted to the Faculty of the Department of Pathology
Graduate Program of the Uniformed Services University of
the Health Sciences in partial fulfillment of the
requirements for the degree of
Master of Science 1990

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DEDICATION

To my mother and father, for their love,

    support and their dreams.

To my wife Xuan, for her support, her

    patient, her sacrifices and her love.

To my daughter Vicky,who some day will

    understand her Dad's love.

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INTRODUCTION

D. radiodurans is remarkable for extreme resistance to UV
radiation, ionizing radiation, and many chemical agents that
damage DNA (for review see Moseley, 1983). Two independent,
functionally overlapping excision repair pathways for UV damage
have been deduced by Moseley and coworkers on the basis of
substantial in vivo evidence, including mutational analysis of
sensitivity to UV and chemical agents, production of single-
strand incisions, removal of pyrimidine dimers from chromosomal
DNA, and analysis of DNA degradation products (Evans and Moseley,
1983; Moseley and Evans, 1983). One excision repair pathway is
thought to be initiated by UV endonuclease-α, which requires a
functional mtcA gene, and the other by UV endonuclease-β, which
requires functional uvsC, uvsD, and uvsE genes. Only UV
endonuclease-β has been detected in vitro and partially
characterized as incising UV irradiated DNA at the same frequency
as the PD-glycosylase from Micrococcus luteus (Evans and Moseley,
1985).

The mutational inactivation of UV-endonuclease-β alone
(uvsC⁻, uvsD⁻, or uvsE⁻) produces only slight sensitization to
UV, while the inactivation of UV endonuclease-α alone (mtcA⁻)
produces no decrease at all in UV survival. However, if both are
mutationally inactivated (i.e., mtcA⁻uvsC⁻, mtcA⁻uvsD⁻, or
mtcA⁻uvsE⁻), UV-sensitivity is dramatic and there is no incision

of chromosomal DNA or removal of pyrimidine dimers (Evans and Moselely, 1983; Moseley and Evans, 1983).

Although inactivation of UV endonuclease-α alone (mtcA⁻uvsCDE⁺) does not produce UV sensitivity, it has been shown to result in sensitivity to several chemical agents that generate DNA adducts, including ethyl methanesulphonate, N-acetoxy-N-2-acetylaminofluorene, and, most strikingly, mitomycin C (Moseley and Copland, 1978; Tempest and Moseley, 1980). Other than modest sensitivity to UV, and a diminution in the rate of pyrimidine dimer removal, mutants in UV endonuclease-β alone (uvsC⁻, uvsD⁻, or uvsE⁻) have no other known phenotypic properties, and are resistant to alkylating agents and mitomycin C (Moseley and Evans, 1983). Neither endonuclease appears to play a direct role in recombination, as single and double mutants are wild-type with respect to transformation efficiency and show the extreme resistance to ionizing-radiation typical of D. radiodurans (Moseley and Evans, 1983).

The introduction of characterized heterologous repair-related genes to repair deficient strains of D. radiodurans is a potentially useful avenue of investigation. In this research project I have expressed bacteriophage T4 pyrimidine dimer-DNA glycosylase in D. radiodurans wild-type and excision repair mutants, and determined the resulting effects on UV survival. Previous investigators have been successful in introducing several drug resistance-determinants and lacZ to D. radiodurans (Smith et. al., 1988; Lennon and Minton, 1990), but this is the

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first time a heterologous DNA repair-related gene has been expressed in this organism.

Numerous attempts to express heterologous genes in D. radiodurans have been unsuccessful (Mackay et al., 1985), a major obstacle being the inability of D. radiodurans to recognize promoters effective in other better characterized bacteria (Smith et al., 1988, 1989, 1990a, 1990b). Consequently, we elected to clone the denV gene into a "duplication insertion" vector (pS11) previously described in this laboratory (Smith et al., 1988) (Fig. 1). This vector is composed of a D. radiodurans chromosomal fragment covalently linked to a heterologous kanamycin-resistance determinant (aphA in the E. coli plasmid pMK20) that is very weakly expressed in D. radiodurans. Upon transformation, recombination of the D. radiodurans fragment in the vector with its corresponding homologous chromosomal sequence yields integration of the heterologous DNA flanked by a direct repeat of the host sequence (Fig. 1). The presence of flanking direct repeats fosters gene amplification (presumably by uneven homologous recombination of daughter chromosomes), and kanamycin selection produces up to 50 tandem copies per chromosome of the amplification unit (AU; Janniere et al., 1985), the AU consisting of the heterologous DNA plus flanking host sequence (Smith et al., 1988). As reported here, inclusion of denV in the AU permits expression of this gene by virtue of high copy number, without a known D. radiodurans promoter immediately adjacent.
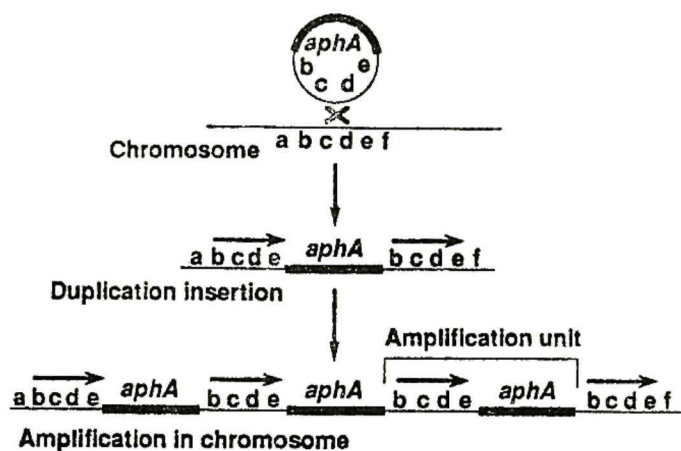
Figure 1. <u>Duplication insertion and amplification.</u>

The thick segment is the <u>E. coli</u> plasmid pMK20, which contains the kanamycin resistance (Km^R) determinant <u>aphA</u> (aminoglycoside-3'-phosphotransferase type I). Thin segment within pMK20 is a cloned fragment of <u>D. radiodurans</u> DNA; its sequence is represented by <u>bcde</u>. The homologous sequence within the chromosome of the <u>D. radiodurans</u> recipient is also shown. Upon recombination (producing duplication insertion) <u>bcde</u> is present as a direct repeat flanking pMK20, indicated by the horizontal arrows. Subsequent gene amplification with continued growth on kanamycin-containing agar yields many copies of the sequence indicated as "Amplification unit."

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BACKGROUND

DNA Damage

Spontaneous alteration in the chemistry of DNA bases

Tautomeric shifts: Each of the bases in DNA can spontaneously undergo a transient rearrangement of bonding, termed a tautomeric shift, to form a structural isomer of the base. Formation of the tautomer of any base alters its base-pairing properties (Friedberg, 1985). This will cause mispairing, and misincorporation may occur during DNA replication in the daughter strands.

Deamination of bases: Three of the four bases normally present in DNA (cytosine, adenine and guanine) contain exocyclic amino groups. The loss of these groups (deamination) occurs spontaneously in pH-acid temperature dependent reactions and results in the conversion of the affected bases into uracil, hypoxanthine and xanthine, respectively. Some of these products of deamination are potentially mutagenic, since during semiconservative synthesis of DNA they constitute miscoding lesions that result in altered base pairs in the genome (Lindahl, 1979).

Loss of bases--depurination and depyrimidization: The most abundant loss of purines and pyrimidines from DNA occur at acid pH; however, depurination occurs at appreciable rates in neutral or alkaline pH. Based on experimentally determined rates of depurination at pH 7.4, it may be calculated for a

mammalian cell that grows with a generation time of 20 hr, 12,000 purines should be lost from the DNA in each cell generation and consequently must be repaired (Lindahl and Nyberd, 1972).

Chemical damage to DNA

Alkylating agents: Alkylating agents are electrophilic compounds with affinity for nucleophilic centers in organic macromolecules. These agents can be either monofunctional or bifunctional. The monofunctional agents have a single reactive group and thus covalently interact with single nucleophilic centers in DNA. The sites of reaction with DNA for many monofunctional alkylating agents, such as acetylaminofluorine, include the following: in adenine, N^1, N^2, N^3, N^6 and N^7; in guanine, N^1, N^2, N^3, N^7 and O^6; in cytosine, N^3, N^4 and O^2 and in thymine, N^3, O^2 and O^4. Bifunctional agents have two reactive groups, and each molecule is potentially able to react with two different nucleophilic centers in DNA. If these sites are situated on the same polynucleotide chain of a DNA duplex, the reaction product is referred to as an intrastrand cross-link. If the two sites are on opposite polynucleotide strands, interstrand cross-links result. The prototypical bifunctional alkylating agent is mitomycin C, which forms both monoadducts and crosslinks. Interstrand DNA cross-links represent an important class of chemical damage to DNA since they prevent DNA strand separation and hence can constitute complete blocks

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to DNA duplication and transcription (Roberts, 1978; Singer, 1982).

Chemicals that are metabolized to electrophilic reactants: A variety of relatively nonpolar compounds undergo metabolic activation to more reactive forms which can interact with nucleophilic centers in DNA. Many of these compounds are potent mutagens and carcinogens. The metabolic activation of these compounds is affected by the induction of specific metabolizing enzymes in the affected cells. The biological function of these enzyme systems is to protect the cells against cytotoxicity effects by converting potentially toxic nonpolar chemicals into water-soluble, excretable forms. Although most of the products of these reactions are harmless, some of them become activated to electrophilic forms that are reactive with nucleophilic centers in organic macromolecules such as DNA. Thus, although these agents are no longer directly cytotoxic, they have been converted into potent genotoxic forms (Miller, 1978: Hiatt, Watson and Winston, 1977).

Metabolic activation of benzo(a) pyrene: Unmodified beno(a)pyrene is an unreactive nonpolar compound with a planar configuration. That configuration facilitates its intercalating between the H-bonded base pairs in duplex DNA. However, some of the products of benzo(a)pyrene metabolism are electrophilic epoxies and the ultimate carcinogenic form of this hydrocarbon is an anti-diol epoxide called 4-7,t-8-

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dehydrase-t-9-10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene. At least one major site of reaction of this product with DNA is the 2-amino group of guanine, which can covalently interact with the C^{10} position of the benzo(a)pyrene anti-diol epoxide (Selkirk, Macleod, Moore, Mansfield, Nikbakht and Dearston, 1982).

Physical agents that damage DNA

Ultraviolet radiation (UV): UV radiation-induced damage results principally from the direct absorption of photons by bases in DNA. DNA damage can also result from wavelengths in the electromagnetic spectrum that are not absorbed directly by bases but are absorbed by other molecular species (sensitizer molecules), which then transfer energy to the bases in DNA. The most abundant UV-induced lesions in DNA are bipyrimidine photoproducts, and of these, about 90% are cyclobutane pyrimidine dimers. When DNA is exposed to radiation at wavelengths approaching its absorption maximum (about 260nm), adjacent pyrimidines become covalently linked by the formation of a four-membered ring structure resulting from the saturation of their respective 5,6 double bonds. The structure formed by this photochemical cycloaddition is referred to as a cyclobutane pyrimidine dimer (Kittler and Lober, 1977; Smith and Hanawalt 1969)(Fig. 2a). The formation of pyrimidine cyclobutane dimers during the irradiation of DNA is a reversible process that can be represented as
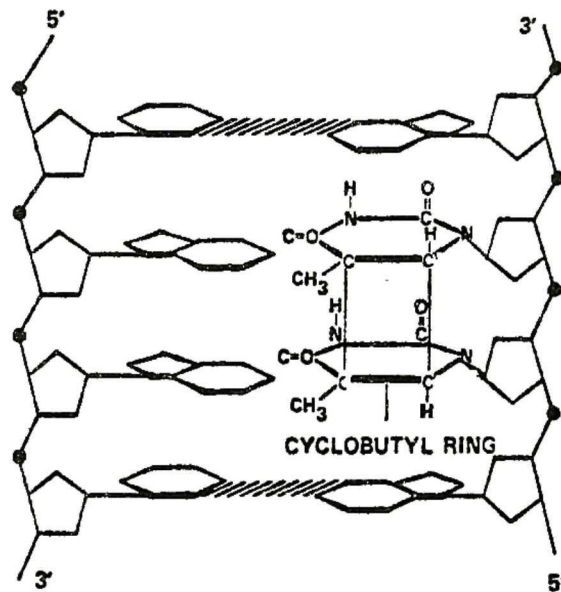
Figure 2a. The cyclobutyl pyrimidine dimer is formed in DNA by the covalent interaction of two adjacent pyrimidines in the same polynucleotide chain. Saturation of their respective 5,6 double bonds results in the formation of a 4-membered cyclobutyl ring linking the two pyrimidines. **from:** Friedberg, E.C. (1985). DNA Repair, Freeman and Co., New York

Py+Py <- - - -> Py<> Py. Since under normal conditions (no dimers in DNA) the equilibrium is shifted to the right, dimer formation is favored over dimer reversal. But at high doses of UV light, the extent of dimer formation reaches a steady-state, which is unaffected by further irradiation. This occurs when dimers are sufficiently abundant such that the rate of dimer formation equals the rate of reversal to pyrimidine monomers (Radany et al, 1981: Gordon et al, 1982).

The major kind of cyclobutane pyrimidine dimers are thymine-thymine dimers, with lesser amount of thymine-cytosine and cytosine-cytosine dimers. The second most abundant bipyrimidine photoproduct is 6-4-[pyrimidin-2'-one]-pyrimidines (6-4 photoproducts) composing about 10% of all bipyrimidine photoproducts (Patrick, 1977; Brash and Hazeltine, 1982). This photoproduct is characterized by a single covalent linkage between the 6 position of the 5' pyrimidine and the 4 position of the 3' pyrimidine (Fig. 2b). The most common 6-4 lesion is between a 5' thymine and 3' cytosine. Unlike pyrimidine cyclobutane dimers, UV radiation (_254nm) serves only to form these lesions and does not reverse them to the original monomers. Consequently, these photoproducts continue to accumulate at high doses of UV until all possible sites are saturated by photoproduct formation or otherwise destroyed. Pyrimidine dimers or 6-4 photoproducts in a template DNA strand block both the progression of transcription and DNA replication. Other UV-induced DNA damage such as monomeric
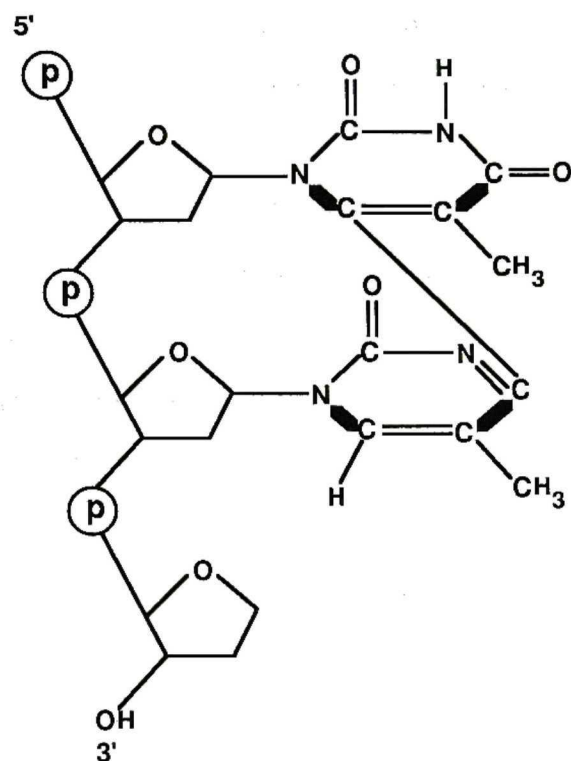
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Figure 2b. Schematic representation of a photoproduct produced by linkage between the C^4 position of one thymine and the C^6 position of the adjacent thymine. This covalent linkage is unstable to hot alkali. In DNA the 3' pyrimidine in such (6-4)lesions is typically cytosine rather than thymine.

base damage with formation of thymine glycol are extremely less common than pyrimidine dimer or 6-4 photoproduct formation (Kittler and Lober, 1977).

Ionizing radiation damage to DNA: Unlike UV radiation, which is preferentially absorbed by nucleic acids, ionizing radiation can cause damage to all cellular components, including DNA. The deposition of energy from ionizing radiation results in the formation of excited nuclei and ionized species. The ionizing radiation damage to DNA has both direct and indirect effects on these molecules. The so called direct effects result from the direct interaction of the radiation energy with DNA. Indirect effects result from the interaction of reactive species formed by the radiation with DNA (Ward, 1975).

DNA Repair

Following damage to DNA, there are a series of cellular responses dedicated to the restoration of the normal nucleotide sequences and of the chemistry of DNA. For instance, after UV induced pyrimdine dimers are generated, unless pyrimidine dimers are removed from DNA prior to replication, affected cells will die. There are several different pathways of DNA repair described in a number of organisms studied to date.

Photoreversal (enzymatic photoreactivation of pyrimidine dimers):

Photoreversal is a light dependent process involving the enzyme-catalyzed monomerization of cyclobutyl pyrimidine dimers. The enzyme activity that catalyzes photoreactivation of dimers is the photoreactivating enzyme of DNA photolyase (Figure 3). DNA photolyase activity has been detected in a large number of plant and animal cell extracts as well as in prokaryotic cells (Rupert, 1975).

Excision repair: The major mechanism for removal of bipyrimidine photoproducts and DNA adducts such as benzo(a)pyrenes is excision repair. This pathway is initiated by an enzyme that recognizes the photoproduct or adduct and produces one or more incisions within it or nearby on the same strand. This initial cleavage is followed by removal of the single stranded fragment of DNA that contains the lesion, follwed by synthesis of a repair patch by a DNA polymerase and ligation of the 3'-hydroxyl of the newly synthesized repair patch to the 5'-phosphoryl of adjacent nondamaged DNA, completing the repair process. The existence of excision repair in most organisms has been deduced by in vivo studies in which repair is allowed to progress for various times following UV-irradiation of cells; the cells are then lysed and the state of DNA and excised products determined (Grossman et al, 1988).

The specificity of excision repair is in the initial step, which requires an enzyme that recognizes the DNA damage and incises DNA in response. Only three such enzyme have been
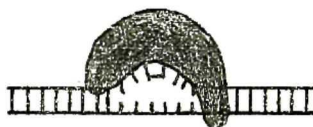
(1) Native DNA

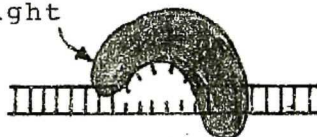(2) Pyrimidine dimer
    in UV DNA

(3) Complex of DNA with
    photoreactivating enzyme

(4) Absorption of light(>300 nm)
Light

(5) Release of enzyme to restore native DNA
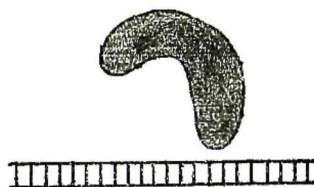
Figure 3. Schematic illustration of the enzyme-catalyzed monomerization of pyrimidine dimers (an example of DNA repair by the reversal of base damage).

well characterized in vitro: the Uvr ABC enzyme of E. coli, the pyrimidine dimer-DNA glycosylase of bacteriophage T4, and the dimer-glycosylase of Micrococcus luteus.

Excision repair in E. coli is governed by the uvr A, uvr B and uvr C genes. Mutations in any one of these genes renders the cell sensitive to a wide variety of DNA-damaging agents including UV, nitrous acid, and mitomycin C. The uvr A, uvr B and uvr C genes have been isolated from E. coli and cloned into various recombinant plasmids through their ability to restore UV resistance to the appropriate UV-sensitive E. coli mutants after transformation with cloned DNA. The three genes were found to code for polypeptides of molecular weights 114000 (Uvr A), 8400 (Uvr B) and 70000 (Uvr C)(Sancar, et al, 1981a, 1981b). The E. coli Uvr ABC enzyme recognizes distortion of the DNA sugar-phosphate backbone caused by dimers, 6-4 photoproducts, and a wide variety of chemical adducts (Franklin and Hazeltine, 1984; Rupp et al, 1982). The predominant UV endonuclease activity of these enzymes is ATP dependent and Mg^{2+} requiring. Uvr ABC proteins function as a complex. Single proteins or mixtures of any two of the three Uvr proteins do not result in incision of DNA containing the base damage. The Uvr ABC enzymes produce two single-stranded endonucleolytic incisions of the DNA strand that contains the damage, one incision seven nucleotides 5' to the site of the

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damage, and the other three to four nucleotides 3' of the same damage site (Fig. 4)(Grossman et al, 1980)

The dimer-glycosylases of bacteriophage T4 and Micrococcus luteus are very similar: they specifically recognize only dimers, and not 6-4 photoproducts or chemical adducts, and have been shown in vitro to produce two sequential cleavages: first, a glycosylic cleavage between the 5' pyrimidine member of the dimer and its corresponding ribose, leaving an apyrimidinic site, and second, a cleavage of the apyrimidinic site by an apurinic/apyrimidinic (AP) endonuclease activity physically associated with the dimer-glycosylase, producing an endonucleolytic cleavage of the sugar-phosphate backbone at the site of the dimer (Fig. 5) (Radany, 1981; Lindahl, 1979).

Recombinational repair: The existence of a form of repair which depends on recombinational exchanges was first suggested by Howard-Flanders and Boyce (1964) and was later supported by the observation that recombination deficient (recA⁻) strains of E. coli were UV sensitive, but excision proficient. Also, it was found that a recA uvrB double mutant was more UV sensitive than strains carrying either single mutation which suggested that the recA dependent repair pathway was distinct from the uvrABC dependent pathway. The steps of recombinational repair are as follows. After UV damage to the DNA strands, replicative bypass of the template damage results in a normal sister duplex DNA molecule and one containing a gap

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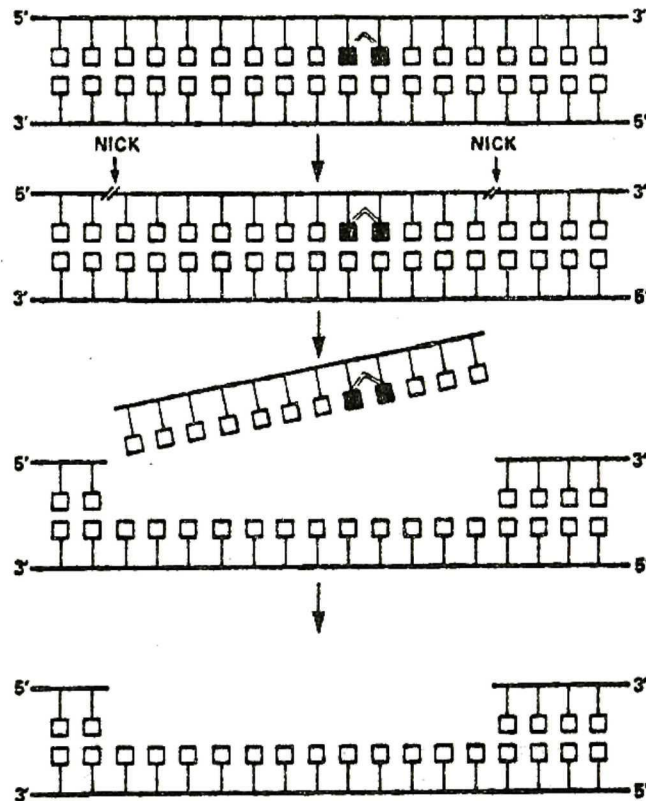
Figure 4. Postulated model of coordinated incision and excision of base damage, such as pyrimidine dimers by the E. coli uvrABC enzyme. The enzyme catalyzes the hydrolysis of phosphodiester bonds on either side of a dimer. The nick 5' to the dimer is seven nucleotides upstream from the dimer, whereas that 3' to the dimer is three or four nucleotides downstream. Thus, an oligo-nucleotide fragment about 12 nucleotides in size is released from the DNA, leaving a gap in the DNA duplex. **from:** Sancar, A. and Rupp, W.C. (1983), Cell **33:** 249.
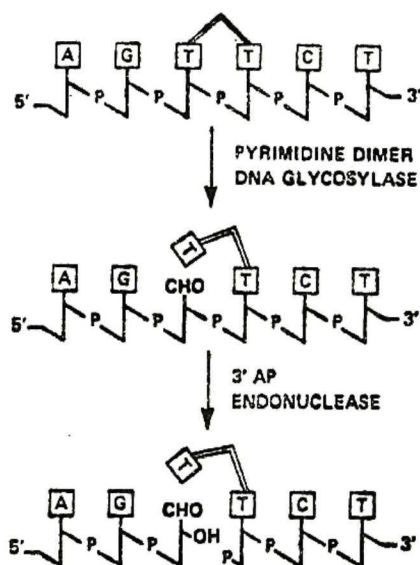
Figure 5. Cleavage of a single N-glycosyl bond in a pyrimidine dimer by a PD DNA glycosylase followed by the cleavage of the sugar-phosphate backbone 3' to the AP site.

opposite the dimer. A nonreciprocal recombinational event
fills the gap in this duplex, leaving a temporary gap in the
isopolar strand of the other sister duplex which is filled in
by repair synthesis using the normal complementary strand as a
template (Fig. 6) (Evans, 1984).

The postulated mechanism of recombination exchange
requires an homologous DNA duplex in the vicinity of the
daughter strand gap. Exchange of DNA single strands then
occurs via a sequence of steps involving breakage of DNA single
strands, heteroduplex formation, strand exchange and re-
ligation of DNA strands. The RecA protein has been directly
implicated in this process _in vivo_ and _in vitro_ where it has
been shown to promote homologous pairing of single strands to
duplex DNA, the pairing of duplex DNA to daughter strand gaps,
the transfer of DNA single strand to form a heteroduplex and
the pairing of two DNA duplexes into a four-strand
heteroduplex. The Rec A protein is considered a key protein in
recombination exchange repair (Cox, 1982; Howard-Flanders,
1968).

Regulation of DNA repair (the SOS phenotype): The
exposure of exponentially growing E. coli to DNA-damaging
agents such as UV radiation or mitomycin C results in the
induction of a wide range of cell responses collectively
referred to as the "SOS" response (Kenyon and Walker, 1980).
These include prophage induction, division delay, inhibition of
the degradation of the host DNA, aberrant re-initiation of DNA

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Figure 6. Current model of the exchanges in DNA associated with postreplication recombination repair. **A:** The replication fork approaches a pyrimidine dimer. **B:** A daughter strand gap is created due to a failure to replicate across a pyrmidine dimer. **C:** A break is introduced in a parental DNA strand. The 3'-OH end is assimilated into the daughter strand gap by the <u>recA</u> protein. **D:** The <u>recA</u> protein drives the strand transfer over the dimer and causes reciprocal strand transfer via a Holliday structure. The pyrimdine dimers can then be repaired via excision repair. **E:** Repaired DNA. Newly synthesized DNA is in white and parental DNA is in black. **from:** Evans, D.M. (1984). Ph.D. Thesis, Edinburgh University.

replication, long patch repair synthesis of DNA during excision repair and shutoff of cellular respiration. The central feature of control of the SOS response is the Lex A protein which is a repressor of its own synthesis and the synthesis of nine or more other gene products including that of the recA gene. When DNA is damaged, a signal, possibly the production of short oligonucleotides or single-stranded DNA or the appearance of single-strand gaps, activates the RecA protein which has in addition to its DNA strand exchange activity, protease activity. This protease activity cleaves the Lex A protein thereby inactivating it and allowing synthesis of the gene products under Lex A repression (which includes its own synthesis). In the induced state derepression of the recA gene protease results in the production of large amounts of Rec A protein so that more protease can be generated to continue degrading Lex A repressor protein. Other genes under Lex A control are also de-repressed. When the inducing signal disappears (perhaps by repair of the single-strand gap), the level of active protease drops, lex A repressor accumulates and the genes under Lex A control are once again repressed (Little and Mount, 1982; Howard-Flanders, 1981; Little et al, 1980)(Figure 7).

Deinococcus radiodurans

Deinococcus radiodurans, a gram-positive, nonsporulating, red pigmented bacterium, is the type species of a small group of bacteria which are characterized by extreme

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UNINDUCED STATE

lexA REPRESSOR   TO OTHER GENES CONTROLLED BY lexA REPRESSOR

PROTEIN

lexA GENE   *recA* GENE   INDUCIBLE   MESSENGER
                              GENE   RNA

OPERATOR

REPRESSOR
ACCUMULATES

DROP IN
PROTEASE LEVEL

DROP IN LEVEL
OF SIGNAL

DNA REPAIRED

DNA
DAMAGE

INDUCING
SIGNAL

recA PROTEASE
ACTIVATED

lexA REPRESSOR
CLEAVED

PYRIMIDINE DIMER

ACTIVATED recA PROTEASE
CLEAVED lexA REPRESSOR

lexA REPRESSOR

recA PROTEIN

lexA GENE   *recA* GENE   INDUCED
                              GENE
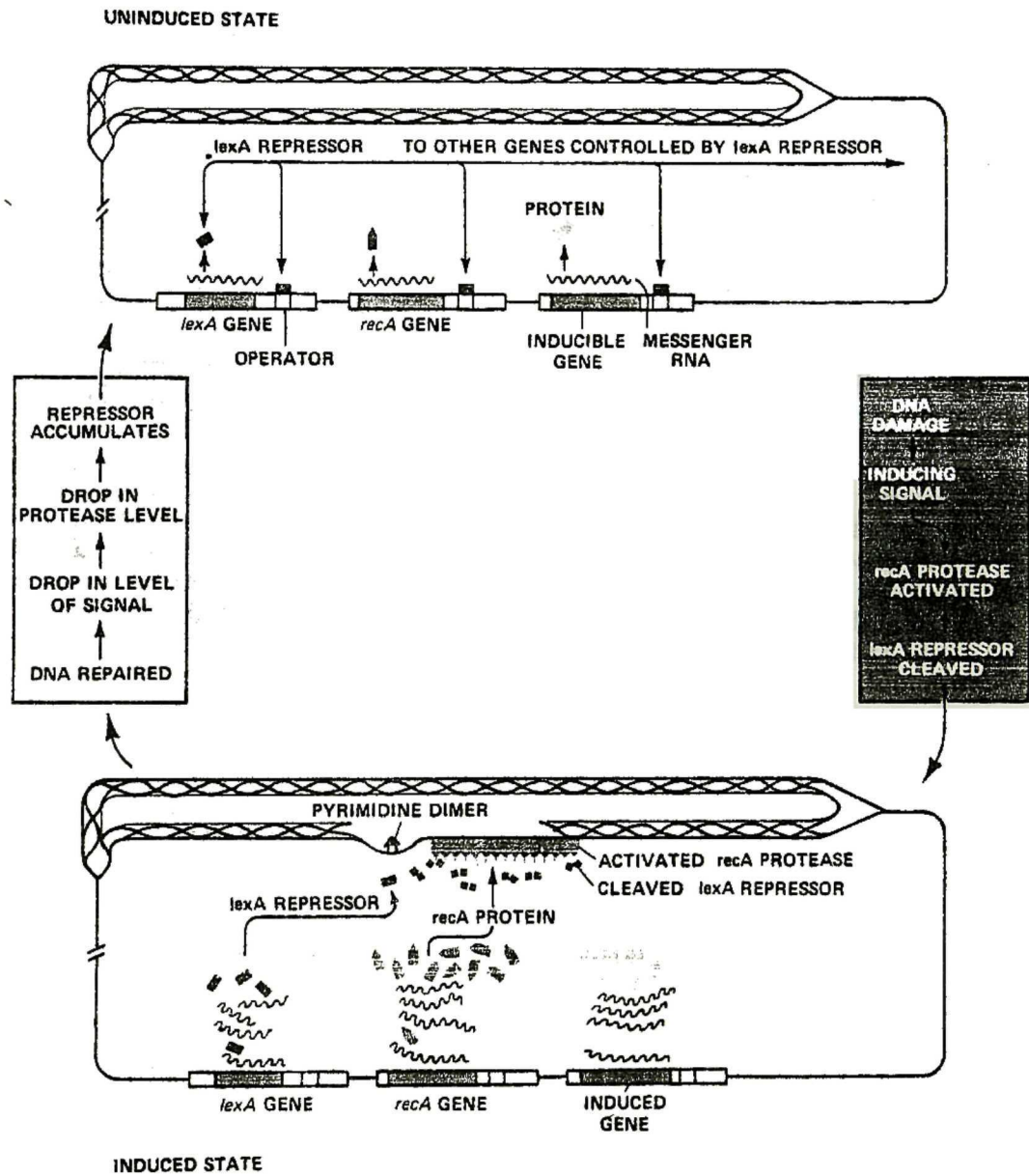
INDUCED STATE

Figure 7. For legend see next page.

Figure 7. Diagrammatic representation of the mechanism
by which the lexA-recA regulon is regulated. In the
uninduced state (top) lexA repressor protein
constitutively expressed in low amounts is bound to the
lexA operator and to the operators of the recA gene and
other genes under lexA control. These genes are still
able to express small amounts of the proteins the encode:
thus, there is some recA protein constitutively present
in uninduced cells. Following DNA damage (e.g. the
presence of a pyrimdine dimer near a replication fork
after induction by UV radiation), existing recA is
activated to a form that is required for the generation
of an active protease that cleaves lexA repressor,
perhaps by binding to the single-strand DNA in the gaps
created by discontinuous DNA synthesis past the dimers
(bottom of figure). In the induced state (bottom)
derepression of the recA gene results in the production
of large amounts of recA protein so that more protease
can be generated to continue degrading lexA repressor
protein. Other genes under lexA control are also
derepressed, although not necessarily with identical
kinetics. When the inducing signal disappears (perhaps
by repair of the single-strand gap), the level of active
protease drops, lexA repressor accumulates and genes
under LexA control are once again repressed. **from:**
Little, J.W. and Mount, D.W. (1982), Cell **29**:11.

resistance both the lethal and mutagenic effects of ionizing and ultraviolet (UV) radiation. There is no loss of viability with doses of up to 500 Krad or 500 Jm^{-2} of ionizing or UV radiation, respectively; and this has made these organisms particularly useful for studying aspects of DNA damage and repair in populations in which every member is a survivor (Moseley, 1983).

Deinococcus radiodurans is normally grown in TGY broth (Bactotryptone 0.5%; glucose 0.1%; yeast extract 0.3%; in distilled water) at 30^0C with shaking or aeration. Under such conditions, it has a doubling time of about 80 min. The DNA of D. radiodurans is responsible for between 1.5 and 2.0% of its dry weight, has a G+C content of 66-68% and is unusual in that it completely lacks methylated bases (Moseley, 1983).

The ionizing and UV radiation survival curves for D. radiodurans have large shoulders, an indication that the bacteria can initially absorb radiation energy with no loss of viability, i.e. it can accumulate sublethal damage followed by an exponential loss of viability (Moseley, 1983). Although Deinococcus radiodurans is extremely resistant to both the lethal and mutagenic effects of a variety of DNA-damaging agents, it has been shown to possess only two mechanisms for the repair of UV-irradiated DNA: excision repair and recombination repair (Moseley and Copland, 1978). While all other organisms that have been studied contain only a single enzyme capable of incising DNA in response to the presence of

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pyrimidine dimers, <u>D. radiodurans</u> is remarkable for having two
different and independent incisional enzymes that recognize
dimers and cleave DNA in response. Either enzyme alone can
incise dimer-containing DNA, and both enzymes must be rendered
inactive by mutation in order to produce a fully UV-sensitive,
DNA incisionless phenotype (Moseley and Evans, 1983). UV
endonuclease-alpha requires expression of the <u>mtcA</u> and <u>mtcB</u>
loci, while UV endonuclease-beta requires expression of the
<u>uvsC, uvsD,</u> and <u>uvsE</u> loci. UV endonuclease-alpha is required
for wild-type resistance to mitomycin C. On the other hand,
strain 302(<u>mtcA</u>⁻ <u>uvsE</u>⁺) is fully resistant to UV by virtue of
the presence of UV endonuclease-beta. Cells deficient in UV
endonuclease-beta (strain HY4000, <u>mtcA</u>⁺ <u>uvsE</u>⁻) are fully
resistant to mitomycin C damage, due to the presence of UV
endonuclease-alpha, but are partially sensitive to UV.
Consequently, it may be concluded that UV endonuclease-alpha
recognizes UV damage, but not with the same effectiveness as UV
endonuclease-beta. Cells deficient in both endonucleases
(strain 78 <u>mtcA</u>⁻ <u>uvsE</u>⁻) are very sensitive to both mitomycin C
and UV (Moseley and Copland, 1978; Tempest and Moseley, 1980;
Evans and Moseley, 1983; Al-Bakri et al, 1985).

exam   UV endonuclease-alpha appears to recognize deformation
of the DNA backbone, rather than the dimer <u>per se</u>, since it
incises DNA not only in response to dimers but also a variety
of chemical adducts in DNA, including mitomycin C. The wide
substrate range of this enzyme suggests a similarity to the <u>E.</u>

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coli Uvr ABC enzyme. The substrate range of D. radiodurans UV endonuclease-beta is not known, but includes dimers. The activities of UV endonuclease-alpha and UV endonuclease-beta have been largely deduced by phenotypic characterization of D. radiodurans strains that are either singly or doubly mutant in the above-cited loci believed to encode these endonucleases. Physical characterization of these enzymes has not proceeded as far. While UV endonuclease-alpha has not been detected in extracts of D. radiodurans, initial characterization of UV endonuclease-beta has revealed a protein of 36,000 daltons that cleaves UV-irradiated DNA. The site of cleavage has not been determined; however, UV endonuclease-beta has been shown not to be a dimer-glycosylase (Evans and Moseley, 1985; 1988).

While the steps in excision repair in E. coli are understood as noted above, it is not possible to extrapolate that model to D. radiodurans, because its repair capacity distinguishes it from all other known organisms. It has been described in the Guinness Book of Records as the "toughest bacterium in the world". They show no loss of viability up to doses of ionizing or UV radiation, that would reduce the survival of any other organism on the order of 10 logs. For example, following exposure to ultraviolet or ionizing radiation, D. radiodurans is able to repair 70,000 UV-induced thymine containing pyrimidine dimers per genome or 18,000 single-strand breaks and over 200 double-strand breaks per chromosome (Moseley, 1983). The presence of more than a few

double-strand breaks is lethal in other organisms.
Consequently, it cannot be assumed that UV endonuclease-alpha
and UV endonuclease-beta behave similarly to repair nucleases
in other organisms. It, therefore, remains to be determined
whether incision is near or far from dimers, whether the
initial incision of UV-irradiated DNA is single or double
stranded; and, if single stranded, whether the incision is in
the damaged or undamaged strand.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strains of Deinococcus radiodurans used are described in Table 1. D. radiodurans strains were grown at 32^{0}C in TGY broth in a rotating shaker or on TGY plates containing 1.5% agar. TGY medium contained 5 g bactotryptone (Difco), 1 g glucose and 3 g yeast extract (Difco) in 1 L distilled water. TGY agar was made by adding bactoagar to 15 g/L.

Construction of pPG100

Plasmid pPG100 was generated as illustrated in Figure 8. To construct pPG100, a BamHI-SalI fragment from pTACdenV (Chenervet et al., 1986) containing the denV gene was electroeluted from a 5% polyacrylamide gel. Polyacrylamide gel electrophoresis was carried out using a Protean II (Bio Rad) apparatus. Gel dimensions were 20cm x 20cm x 1.5mm. For a 5% acrylamide gel the following solution was made: 5 ml of 10 x TBE buffer. 8.33 ml of 29:1 acrylamide/bisacrylamide, 36.67 ml of water, 500 ul of 10% ammonium persulfate and 25 ul TEMED. The acrylamide gel mix was poured between the plates, the comb was inserted and the gel was polymerized at room temperature for over 30 min. After polymerization of the gel was complete, the comb was removed and the sample wells were rinsed. Once the plates were attached to the gel electrophoresis tank the lower reservoir of the gel tank and the upper buffer chamber

Table 1

Bacterial Strains and Plasmids

Strain or plasmid	Description: Relevant genotype [a]	Source (reference) [b]
Deinococcus radiodurans		
302	*mtcA⁻ uvsE+*	(Moseley & Evans, 1983)
HY1001	302ΩpS11; *mtcA⁻ uvsE+ aphA+*	302 x pS11
HY1002	302ΩpPG100; *mtcA⁻ uvsE+ aphA+ denV+*	302 x pPG100
HY2000	*mtcA+ uvsE+*	302 x pUE58
HY2001	HY2000ΩpS11; *mtcA+ uvsE+ aphA+*	HY2000 x pS11
HY2002	HY2000ΩpPG100; *mtcA+ uvsE+ aphA+ denV+*	HY2000 x pPG100
UVS78	*mtcA⁻ uvsE⁻*	(Moseley & Evans, 1983)
HY3001	78ΩpS11; *mtcA⁻ uvsE⁻ aphA+*	UVS78 x pS11
HY3002	78ΩpPG100; *mtcA⁻ uvsE⁻ aphA+ denV+*	UVS78 x pPG100
HY4000	*mtcA+ uvsE⁻*	UVS78 x pUE58
HY4001	HY4000ΩpS11; *mtcA+ uvsE⁻ aphA+*	HY4000 x pS11
HY4002	HY4000ΩpPG100; *mtcA+ uvsE⁻ aphA+ denV+*	HY4000 x pPG100
Escherichia coli		
DH5α	F⁻ *recA1*	Bethesda Research Laboratories

Table 1 (cont.)

Plasmids

pUE58	pAT153 *EcoRI*::5.6 kb *D. radiodurans EcoRI* chromosomal fragment containing *mtcA* gene	(Al-Bakri *et al.*, 1985)
pS11	*aphA* (Km^R); 12.5 *EcoRI* fragment of *D. radiodurans* chromosomal DNA in pMK20	(Smith *et al.*, 1988)
pTACdenV	YCp50 with *denV* on 480 bp *Bam* HI-*Sal* I fragment. *tac* promoter upstream of *Bam* HI site.	(Chenevert *et al.*, 1986)
pPG100	pS11 *Dra* I::YCp50 *Bam* HI-*Sal* I *denV*-containing fragment	Blunt end ligation

[a] Many strains may be thought of as having insertions of a plasmid which contains both chromosomal and heterologous sequences and are so designated in accordance with convention (Novick *et al.*, 1976). For example, *D. radiodurans* 302 derivative HY1001 has an insertion of pS11 and so is described as 302ΩpS11.

[b] Strains or plasmids for which a literature reference is not noted were constructed during this study. Transformations are noted as "recipient x donor DNA." pS11 and pPG100 *D. radiodurans* transformants were selected on TGY agar containing 5 μg/ml kanamycin sulfate. Strains 302 and UVS78 carry the *mtcA$^-$* mutation and are consequently sensitive to mitomycin C. pUE58, which contains the *mtcA+* gene, was used to transform these strains to mitomycin C resistance with a selective mitomycin C concentration of 0.03 ug/ml in TGY agar.
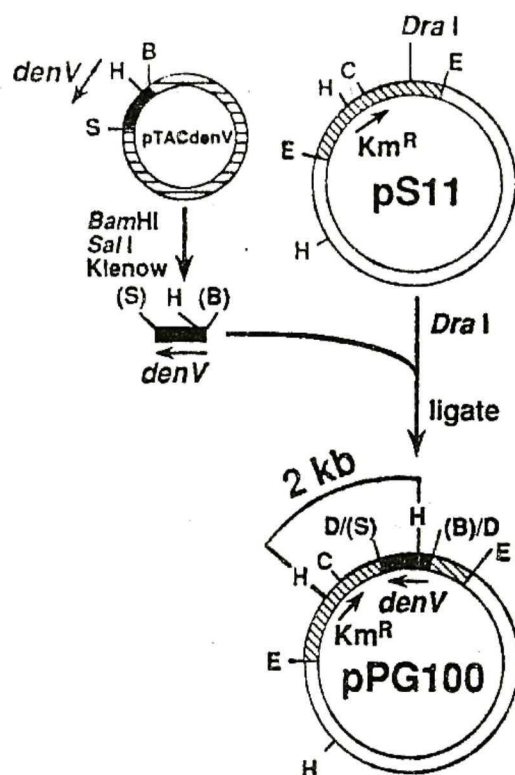
Figure 8. Construction of pPG100. pPG100 was constructed as described in **Materials and Methods.** pMK20: diagonally hatched; <u>D. radiodurans</u> sequence: open. 480 bp <u>denV</u>-containing fragment: closed; Vector sequences of pTACdenV: horizontally hatched. Restriction enzymes: **H** - <u>Hin</u>dIII; **B** - <u>Bam</u>HI; **S** - <u>Sal</u> I; **E** - <u>Eco</u>R1; **C** - <u>Cla</u> I; **(B)** - 5'-protruding end produced by <u>Bam</u> HI cleavage, blunt-ended with Klenow fragment; **(S)** - 5'-protruding end produced by <u>Sal</u> I cleavage, blunt-ended with Klenow fragment. **(B)/D** - Blunt end ligation of (B) with <u>Dra</u> I cleaved site. **D/(S)** - Blunt end ligation of (S) with <u>Dra</u> I cleaved site. Insertion of the <u>denV</u>-containing fragment introduces an additional <u>Hin</u>dIII site. In pPG100 the <u>Hin</u>dIII restriction fragments are 9 kb, 6 kb, and 2 kb. Arrows indicate direction of transcription of <u>denV</u> and <u>aphA</u>.

were filled with 1 x TBE buffer. The DNA samples and markers were loaded. The gel was run at 80V for 3 hr and stained for 30 min in 0.5 ug/ml ethidium bromide to locate the DNA fragments. The DNA band of interest was cut out of the gel, placed into a dialysis bag (M.W.C.O. 10,000) containing 500 ul of 0.5 x TBE buffer. The bag was placed in a horizontal electrophoresis apparatus containing 0.5 x TBE buffer. Electrophoresis was carried out at 50V for 2 hr. The direction of the current was reversed for 30 sec at the same voltage and the solution contents of the bag containing the DNA was transferred to an Eppendorf tube. The DNA was precipitated using 0.1 vol of 3M sodium acetate and 2 vol of ethanol.

The 5' protruding ends of this fragment were filled in using the Klenow fragment of DNA polymerase I (BRL). The Klenow fragment retains the DNA polymerase and the 3'--> 5' exonuclease of E. coli DNA polymerase I, but lacks the 5'-->3' exonuclease activity, making it the ideal enzyme to fill in 5' overhangs. The reaction conditions used to blunt-end the BamHI-SalI fragment were as follows: 20 ul total reaction volume, 2 ug of DNA, 1 ul of 0.5mM dNTP (0.5mM each of the 4 dNTP's) and 2 ul of REreact buffer (BRL), 14 ul of H_2O, 3U of Klenow fragment with incubation at room temperature for 30 min. The reaction was stopped by heating to 75^{0}C.

Plasmid pS11 (Smith et al., 1988) was digested with DraI and the 5' phosphate from both ends of the linear DNA were removed with bacterial alkaline phosphatase (BAP) to prevent

recircularization of the plasmid. BAP catalyzes the hydrolysis of 5'-phosphate residues from DNA. The reaction conditions of BAP enzyme treatment was as follows: 50 ul total reaction volume, 50mM Tris-HCl, pH 8.0, 4 ug DNA, 0.1 U BAP. Incubation was carried out at 60^0C for 60 min. The reaction was extracted twice with phenol, once with chloroform and the DNA was precipitated with ethanol and sodium acetate.

Plasmid pS11 was ligated with the denV gene containing fragment from pTACdenV using T4 DNA ligase. T4 DNA ligase catalyzes the formation of phosphodiester bonds between juxtaposed 5' phosphate and 3'-hydroxyl termini. The reaction conditions were as follows: 50 ul total reaction volume, 40mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10mM DTT, 1 ug DNA, 0.5mM ATP, 50 ug/ml BSA and 1 U T4 DNA ligase. Incubation was at 15^0C overnight.

Transformation of E. coli with DNA

The ligation mix of pS11 and the denV gene containing fragment from pTACdenV was used to transform E. coli according to the method of Okayama and Berg (1982). A single colony of E. coli (DH5 alpha) was inoculated into 10 ml LB medium pH 7.5 (10 g tryptone, 5 g yeast extract, 5 g NaCl and water to 1 liter) and allowed to grow overnight. One ml of this culture was added to 50 ml LB medium and grown to an O.D.$_{560}$ of 0.4 (approximately 90 min). Cells were centrifuged for 5 min at 3200 rpm, 4^0C. The pellet was resuspended in 5 ml ice cold LB + 50 mM $CaCl_2$ + 5% glycerol, aliquoted into prechilled, sterile

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polypropylene tubes and frozen immediately at -70^0C. Just before transformation, the competent cells were thawed slowly on ice. Approximately 10 ng of DNA from the ligation mix were added to 100 ul of competent cells. The samples were kept on ice 30 min, heat shocked at 42^0C for 2 min, and diluted with 900 ul of LB. After a 60 min incubation, 100 ul of this mix was plated on LB plates containing 25 ug/ml of kanamycin. E. coli colonies resistant to kanamycin (the drug resistance determinant in pS11) were screened by colony hybridization to detect the presence of the denV gene.

Colony hybridization

Colony hybridization (Grunstein and Hogness, 1975) is a rapid and effective technique for detecting recombinant sequences. Kanamycin-resistant transformants of E. coli were picked and streaked in 1 cm squares in Kanamycin containing LB plates in order to orient them for later identification. After overnight growth, an S&S nitrocellulose membrane was placed on the surface of the plates. The colonies were transferred for approximately 5 min. The membrane was then lifted from the plates and placed on two pieces of S&S GB003 filter paper saturated with 0.5N NaOH for 5 min. It was then neutralized by placing it on two sheets of S&S GB003 paper saturated with 1M Tris-HCl, pH 8.0/1.5M NaCl for 5 min. The membrane was then briefly washed in 2 x SSC, allowed to dry and baked at 80^0C in a vacuum oven. The probe used was the nick-translated BamHI-

SalI fragment from pTACdenV (which contains the denV gene).
Colonies that hybridized to this probe were picked and grown so
as to prepare DNA by the miniprep method and check for the
presence of the denV gene in pS11.

Miniprep of plasmid DNA

Plasmid minipreps were performed using the alkaline
lysis method (Birnboim and Doly, 1979). Five ml of LB
containing 50 ug/ml kanamycin were inoculated with a single
bacterial colony and incubated overnight. The next morning 1.5
ml of the culture was transferred into an Eppendorf tube and
centrifuged for 2 min. The medium was removed and the pellet
was resuspended by vortexing in 100 ul of an ice-cold solution
of 50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH 8.0, 5 mg/ml
lysosome and incubated for 5 min in ice. A freshly-prepared
solution of 200 ul of 0.2N NaOH, 1% SDS was then added. The
sample was mixed and incubated on ice for 5 min. To
precipitate out bacterial proteins, 150 ul of an ice-cold
solution of potassium acetate (pH 4.8) was added (60 ml of 5M
potassium acetate, 1.5 ml of glacial acetic acid and 28.5 ml of
H_2O). The sample was mixed by vortexing gently and centrifuged
for 5 min at 4^0C. The supernatant was transferred to a fresh
tube, extracted by phenol/chloroform and ethanol precipitated
at room temperature. The DNA pellet was resuspended in 50 ul
of TE containing DNase-free pancreatic RNase (20 ug/ml).

Restriction enzyme analysis of one of the plasmid minipreps showed the presence of pPG100 as shown in Figure 8.

Preparation of plasmid DNA

E. coli DH5alpha containing plasmids pS11 and pPG100 were grown in 1 liter LB broth with kanamycin (50 ug/ml) overnight at 37^0C. The bacteria were harvested by centrifugation at 7000 rpm for 7 min at 4^0C, and the pellet resuspended in 50 ml cold TE, pH 8.0, and spun at 7000 rpm for 7 min at 4^0C again. The cells were resuspended in 9 ml of Sucrose-EDTA-Tris (SET) (25% sucrose, 0.05M Tris-HCl and 40mM EDTA). One ml of lysozyme solution was added (10 mg/ml of lysozyme in SET). Incubation was carried out for 5 min and 3.7 ml of cold 0.25M EDTA, pH 8.0 were added. After 5 min on ice, 14.5 ml of Triton solution (40% Triton X-100, 62.5mM EDTA and 50mM Tris-HCl, pH 8.0) were added and incubation continued at room temperature for 10 min. The sample was transferred to 1 x 3-1/2" polyallomer centrifuge tubes and spun at 25,000rpm in an SW28 rotor for 2 hr at 4^0C. The supernatant was transferred to 50 ml tubes and the plasmid DNA was purified by buoyant density centrifugation in cesium chloride gradients. The CsCl gradient were prepared according to the following formula: (1) x ml supernatant; (2) 1/20 x ml ethidium bromide (10mg/ml); (3) xg + 1/10 xg of CsCl. The gradients were centrifuged in sealed polypropylene tubes at 47,000 rpm in a Vti-50 rotor for 18 hr at 18^0C. Two bands were visible after centrifugation when

illuminated with a 302nm light provided by a hand held UV illuminator. The lower band, corresponding to supercoiled covalently closed circular plasmid DNA was removed by piercing the side of the centrifuge tube with a wide-bore syringe needle. Ethidium bromide was removed from the CsCl solution containing the plasmid by repeated extraction against isopropanol which had been saturated with NaCl saturated distilled water. To precipitate the plasmid DNA, one vol of TE (10mM Tris-HCl, pH 8; 1 mM EDTA) and two vol of ice-cold absolute alcohol were added. The plasmid DNA was pelleted after incubation at -70^0C for 1 hr and centrifugation at 3.4k rpm for 15 min at 4^0C. The pellet was washed with 70% alcohol, dried and resuspended in TE.

Transformation of D. radiodurans

Transformation of <u>D. radiodurans</u> was performed as described by Tigari and Moseley (1980). To prepare competent cells, bacteria were grown overnight at 32^0C with vigorous shaking. The overnight culture was diluted 1 in 10 and allowed to grow to O.D. 0.5. Cells were spun at 3000 rpm for 5 min, resuspended in TGY containing 35mM $CaCl_2$ 10% glycerol and immediately aliquoted and stored at -70^0C. To perform the transformation, competent cells were thawed quickly by incubating at 37^0C and then placed immediately on ice. One ug of plasmid DNA was added to 100 ul of cells (approximately 5 x 10^7 cells per ml). Samples were kept on ice for 5 min and then

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incubated at 32^0C with gentle shaking for 90 min. Then 900 ul
of TGY-Ca^{++}) was added to the transformation mix and cells were
allowed to grow for 90 min to several hours for phenotypic
expression. The culture was then diluted and plated on TGY
agar containing kanamycin (5 ug/ml).

Isolation of D. radiodurans genomic DNA

A growth-saturated liquid culture of Deinococcus with
1.5 ml TGY was spun in a microfuge and resuspended in 1 ml of
butanol-saturated phosphate buffer (this removes the lipid
containing wall layers of the cells which prevents proteinase K
from acting). Bacterial pellets were resuspended in 567 ul TE
by repeated pipetting. Thirty ul of 10% SDS and 10 ul of 20
mg/ml proteinase K were added, and the mix incubated 1 hr at
37^0C. Cell wall debris, polysaccharide and remaining proteins
were removed by adding 100 ul of 5M NaCl and 80 ul of CTAB (10%
of hexadecyltrimethyl ammonium bromide in 0.7M NaCl) solution
and incubated for 10 min at 65^0C. The sample was then
phenol/chloroform/isoamyl alcohol extracted two times. High
molecular weight DNA was precipitated by adding 0.6 vol of
isopropanol and centrifuged in a microfuge. The pellet was
washed with 70% ethanol, dried and resuspended in TE.

Agarose gel electrophoresis and hybridization

D. radiodurans genomic DNA was digested with HindIII
and electrophoresed on horizontal 1% agarose gels. Lanes

contained approximately 1 ug of DNA. Agarose (BRL) was boiled in 1 x TBE buffer, cooled to 50^0C and then cast in a gel casting platform. The gel dimensions were 200 x 6 x 240 mm. When set, TBE buffer was added up to the level of the gel surface and samples of DNA added to the wells in the gel. A constant 30 volts was typically applied and the gel was run for about 18 hr.

Once the electrophoresis was completed, the gel was treated as follows: immersed in 0.25N HCl for 10 min at room temperature, soaked in 1.0M NaCl/ 0.5M NaOH 2 x 15 min for denaturation of the DNA and neutralized by soaking in 0.5M Tris-HCl pH 7.4/1.5M NaCl, 2 x 15 min. To transfer the DNA to nitrocellulose membrane filter, three pieces of S&S GB003 gel blot paper was cut 4-6" larger than the gel, saturated with transfer buffer (10 x SSC) and placed on a platform in a tray. A solution of 10 x SSC was added to the tray. A nitrocellulose membrane (saturated with 10 x SSC) was placed on top of the gel, and on top of the membrane three more pieces of 10 x SSC saturated gel blot paper cut to fit the gel, were added. Then, on top of this a 1" stack of S&S GB004 paper was included. The assembly was secured with a light weight and the transfer to the membrane was allowed to continue overnight. To fix the DNA to the membrane, it was baked at 80^0C.

^{32}P-labeled probe was prepared by nick translation using a BRL nick translation kit. Five ul of solution A (no dCTP, containing dATP, dGTP and dTTP); 1 ug of DNA; 10 ul of

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radioactive nucleotide (alpha-^{32}P-dCTP) and 29 ul of H_2O were mixed (total vol was 45 ul). Five ul of DNA polymerase I/DNAse I was added and mixed gently. The sample was incubated at 15^0C for 60 min. Finally, 5 ul of stop buffer (300 mM Na_2EDTA, pH 8.0) was added. To separate the labeled DNA from free nucleotides, the sample was passed through a 0.9 x 15 cm column of Sephadex G-50 Fine equilibrated with TE buffer. Prehybridization in 5 x SSC, 50% formamide, 0.5% SDS, 100 ug/ml low molecular weight DNA was performed to block active sites on the nitrocellulose to which the free probe can adhere non-specifically. Hybridization was performed in hybridization buffer: 5 x SSC, 5 x Denhardt's Solution, 50% formamide, 0.5% SDS, 100 ug/ml denatured low molecular weight DNA, 10% dextran sulfate. The probe was denatured by boiling in TE buffer for 5 min and quenching in ice. Hybridization was carried out for 18 hr at 42^0C. Blots were washed twice in 1 x SSC/0.1% SDS for 5 min at room temperature and twice in 0.1 SSC/0.1% SDS for 15 min at 65^0C. Finally, a Kodak XOMAT-AR film was exposed to the membrane.

Measurement of survival following UV damage

Overnight cultures of D. radiodurans HY2000 and all mutants used were diluted to an O.D. of 0.6 with TGY. One ml was spun down and resuspended in 10 ml of sterile saline. The suspension was irradiated with a 254nm UV light (GE G875) at a dose of 1.00 $Jm^{-2}sec^{-1}$. Aliquots were taken at different times

(corresponding to different doses) and plated on TGY-1.5% agar. Surviving colonies were counted after two days.

Detection of pyrimidine dimer (PD)-DNA glycosylase activity

Release of free thymine following the photoreversal of thymine-thymine dimers was determined essentially according to Bonura et al. (1982). A schematic diagram of the basis for the method can be seen in Figure 10. For the preparation of cell extract, cells were lysed by one passage through a French pressure cell at 0^0c. Ten ug of crude cell extract were incubated with pUC18 which had been previously nick-translated using TTP, $[CH_3-^3H]$- and treated with 2000 J/m^2 in a total volume of 50 ul to generate thymidine dimers. In the presence of T4 PD-DNA glycosylase, the glycosylic bond between the 5' thymine and its sugar is cleaved. After 1 hr incubation at 32^0C in 10mM Tris-HCl pH 8.0 0.10mM EDTA, the reaction was exposed to 12000 J/m^2 for the photoreversal reaction. This produces the remonomerization of the dimer, releasing free thymine. An equal volume of a solution of 0.2M NaCarbonate - 0.2M ZnAcetate was added which precipitates all DNA components except free bases and nucleosides and the reaction was centrifuged 30 min at 12,000 rpm. Counts per minute were determined from an aliquot of the supernatant, reflecting free thymine.

Introduction of the denV gene into the D. radiodurans chromosome.

The denV gene from pTACdenV was inserted into the Dra I site of pS11, yielding pPG100, as described in **Materials and Methods** and Figure 8. The 480 bp denV fragment included the protein coding sequence of the PD-glycosylase, but did not contain the TAC promoter. We have previously found that this strong E. coli promoter is not recognized in D.radiodurans (M.D. Smith and K.W. Minton, unpublished data). The Dra I site was selected as it is part of the pMK20 portion of pS11 that does not interfere with expression of kanamycin resistance or plasmid replication in E. coli, and does not disrupt the Deinococcus sequence via which duplication insertion occurs in D. radiodurans. Plasmids pS11 and pPG100 were introduced to strains 302 ($mtcA^-uvsE^+$), UVS78 ($mtcA^-uvsE^-$), HY2000 ($mtcA^+uvsE^+$), and HY4000 ($mtcA^+uvsE^-$) as described in **Materials and Methods** and Table 1. D. radiodurans transformed with pS11 or pPG100 were selected on kanamycin-supplemented medium.

Southern blots of genomic DNA from pPG100 transformants indicate the presence of the denV gene (Fig. 9). Genomic DNA was digested with HindIII, electrophoresed, blotted, and probed with nick-translated pPG100. The presence of the 2 kb band that hybridize with this probe is due to the additional HindIII site in the denV-containing strains (Fig. 8). Lanes
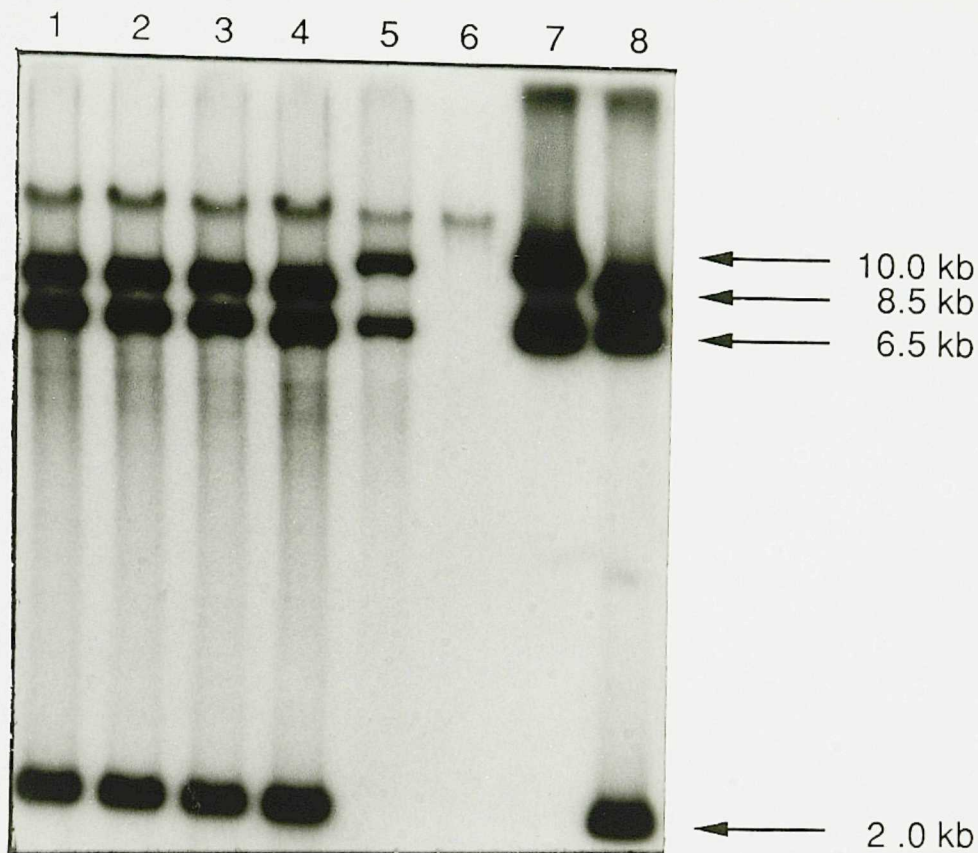
42

Figure 9. Southern blots of genomic DNA from <u>D. radiodurans</u> strains. Genomic DNA was isolated, cleaved with <u>Hind</u>III, electrophoresed on 1% agarose gels and blotted. Each lane contained approx. 1 ug of DNA. The blot was probed with the nick-translated pPG100.
Lanes: 1 - HY2002 (<u>mtcA</u>$^+$<u>uvsE</u>$^+$<u>aphA</u>$^+$<u>denV</u>$^+$);
2 - HY1002 (<u>mtcA</u>$^-$<u>uvsE</u>$^+$<u>aphA</u>$^+$<u>denV</u>$^+$);
3 - HY4002 (<u>mtcA</u>$^+$<u>uvsE</u>$^-$<u>aphA</u>$^+$<u>denV</u>$^+$);
4 - HY3002 (<u>mtcA</u>$^-$<u>uvsE</u>$^-$<u>aphA</u>$^+$<u>denV</u>$^+$);
5 - HY3001 (<u>mtcA</u>$^-$<u>uvsE</u>$^-$<u>aphA</u>$^+$);
6 - UVS78 (<u>mtcA</u>$^-$<u>uvsE</u>$^-$);
7 - pS11; 8 - pPG100;

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containing DNA from non-transformed cells and cells transformed with pS11 do not show this bands.

Detection of dimer-glycosylase activity in extracts of transformed strains.

To determine whether the denV gene product, PD-glycosylase, was expressed when denV was introduced to D. radiodurans by this means, we assayed PD-glycosylase activity in crude extracts of transformants. Of the two glycosylic bonds in a cyclobutane dimer, PD-glycosylase only cleaves the glycosylic bond between the 5' member of the pyrimidine dimer and its base, producing an apyrimidinic site. In addition the T4 PD-glycosylase has an apyrimidinic/apurinic (AP) endonuclease activity that incises the phosphodiester bond 3' to the AP site, producing backbone cleavage. This AP endonuclease activity is easily detected in vitro, but it has yet to be demonstrated in vivo (Radany et al., 1987)

The assay employed is that of Bonura and coworkers (1982), and is based on the release of free thymine following the photoreversal of pyrimidine dimers in ^{3}H-thymine-containing DNA (Fig. 10). After incubation of UV-irradiated DNA with PD-glycosylase, thymidine dimers are subjected to photoreversing irradiation and the reaction mixture is precipitated with zinc acetate and sodium carbonate. This procedure leaves only free bases and nucleosides in the supernatent (Bonura et al., 1982).
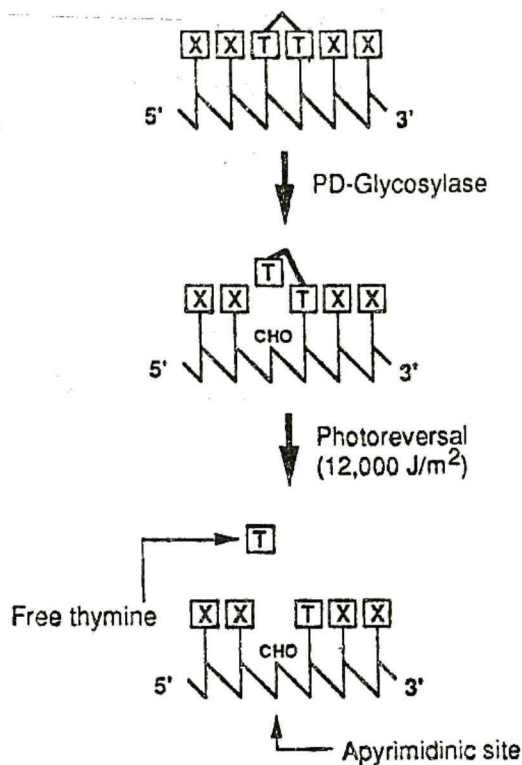
44

Figure 10. Diagram of protocol for assay of PD-glycosylase activity *in vitro.*
Only one strand of a duplex DNA molecule is shown. UV light both induces
and reverses pyrimidine dimer formation. In DNA exposed to high 254 nm UV
fluences, the photosteady-state of thymine in pyrimidine dimers is 7% of total
thymine, even though the theoretical maximum level is much higher. At this
plateau value, the rate of UV-induced dimer formation equals the rate of UV-
induced splitting of dimers to form thymidine monomers. If one
N-glycosylic bond in a thymidine dimer is cleaved by PD-glycosylase the
associated base is in covalent linkage only with its partner. Additional UV
exposure results in dimer splitting, liberating free thymine (Radany *et. al.*, 1981).

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Free thymine was detected at higher than background levels only in strains containing the denV gene (Table 2). This result demonstrates that the denV gene is expressed as an active product in D. radiodurans.

The presence of the denV gene product enhances survival of strain UVS78 (mtcA⁻uvsE⁻) and strain HY4000 (mtcA⁺uvsE⁻).

Figure 11 shows survival curves comparing UV sensitivity of the different strains used in this study. Strains HY2000 (mtcA⁺uvsE⁺), which contains both UV endonucleases, and 302 (mtcA⁻uvsE⁺), which contains only UV endonuclease-β have wild-type resistance to UV. Strain HY4000 (mtcA⁺uvsE⁻), which contains only UV endonuclease-α, has a slightly lower degree of resistance. Strain UVS78 (mtcA⁻uvsE⁻), which is completely incisionless following UV irradiation (Evans and Moseley, 1983), is extremely sensitive to UV damage.

The ability of pPG100 to express denV in Deinococcus as an amplified chromosomal element allowed us to determine whether PD-glycosylase could enhance the UV resistance of these D. radiodurans strains (Fig. 12). Transformation of this plasmid into UVS78 (mtcA⁻uvsE⁻) and HY4000 (mtcA⁺uvsE⁻), resulted in enhanced resistance to UV. On the other hand, transformation with pPG100 had no effect on the UV sensitivity of strain 302 (mtcA⁻uvsE⁺) and strain HY2000 (mtcA⁺uvsE⁺), both of which are wild-type with respect to UV survival.

Table 2

Assay of pyrimidine dimer-DNA glycosylase activity by the zinc acetate/sodium carbonate coprecipitation method

DNA + 2,000 J/m^2 + CRUDE EXTRACT (10 µg protein) + 12,000 J/m^2 [a]

CRUDE EXTRACT	ZnAcetate/NaCarbonate supernatent (50 µl; cpm) [b]
HY2000 (*mtcA+ uvsE+*)	31
HY2001 (*mtcA+ uvsE+ aphA+*)	0
HY2002 (*mtcA+ uvsE+ aphA+ denV+*)	3,414
302 (*mtcA- uvsE+*)	33
HY1001 (*mtcA- uvsE+ aphA+*)	38
HY1002 (*mtcA- uvsE+ aphA+ denV+*)	2,364
HY4000 (*mtcA+ uvsE -*)	0
HY4001 (*mtcA+ uvsE - aphA+*)	20
HY4002 (*mtcA+ uvsE - aphA+ denV+*)	1,932
UVS78 (*mtcA- uvsE -*)	15
HY3001 (*mtcA- uvsE - aphA+*)	0
HY3002 (*mtcA- uvsE - aphA+ denV+*)	1,818
E. coli DH5α/pTACdenV	2,738

[a] Experimental values shown were obtained via this sequence of steps (see text). The only variable was the source of crude extract, which is listed in the left-hand column of the table.

[b] Values shown have background subtracted. Background was determined by using 10 µg of bovine serum albumin instead of 10 µl crude extract (1 µg protein/µl). Background varied with experiment, ranging from 158 to 313 cpm per 50 µl of ZnAcetate/NaCarbonate supernatent.
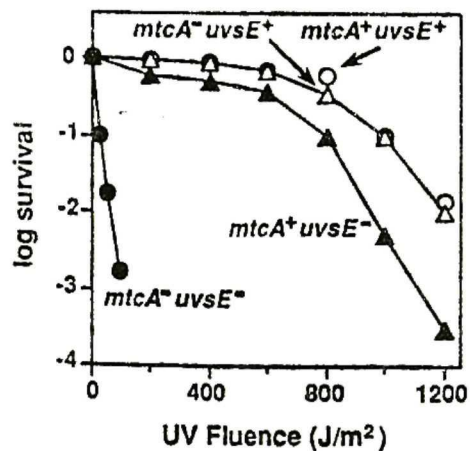
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Figure 11. Survival of <u>D. radiodurans</u> excision repair
mutants. **HY2000** (<u>mtcA</u>⁺<u>uvsE</u>⁺): open circle
302 (<u>mtcA</u>⁻<u>uvsE</u>⁺): open triangle
HY4000 (<u>mtcA</u>⁺<u>uvsE</u>⁻): closed triangle
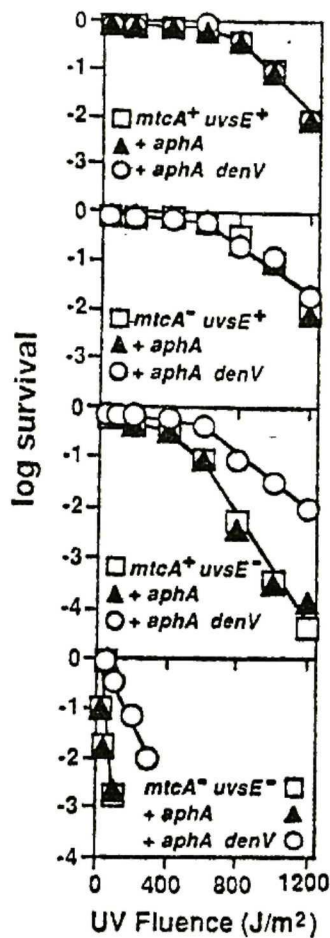**UVS78** (<u>mtcA</u>⁻<u>uvsE</u>⁻): closed circle

Figure 12. Survival of <u>D. radiodurans</u> strains containing the
<u>denV</u> gene. Strains as in Fig. 11 and strains transformed with
pS11 (indicated by "+ <u>aphA</u>") and strains transformed with pS11
containing the <u>denV</u> gene, i.e., pPG100 (indicated by
"+<u>aphAdenV</u>").

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The increase in survival of HY4000 ($\underline{mtcA}^{+}\underline{uvsE}^{-}$) imparted by the presence of PD-glycosylase produced nearly wild-type levels of resistance to UV (Fig. 13, left). Survival of UVS78 ($\underline{mtcA}^{-}\underline{uvsE}^{-}$) is enhanced substantially, yielding a 4-fold decrease in slope (Fig. 13, right); however this increment does not approach the wild-type level of UV-resistance (Fig. 12, top and bottom boxes).
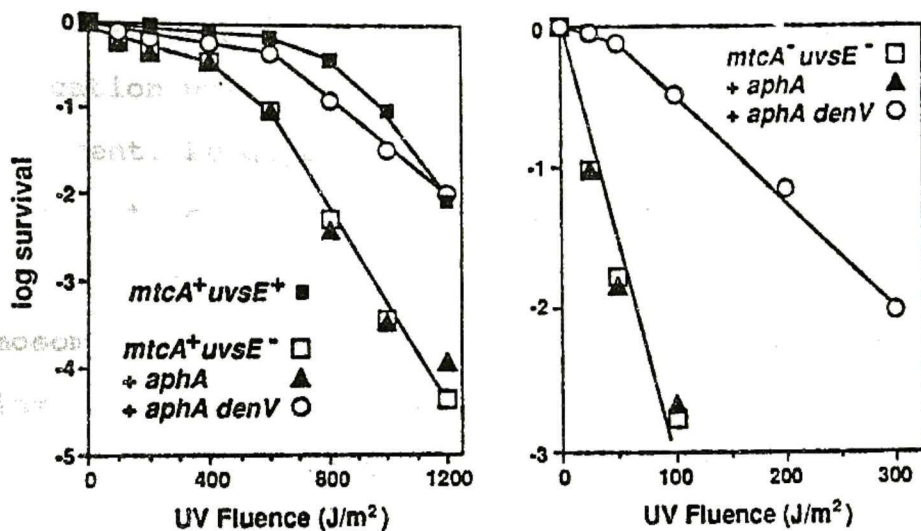
Figure 13. Increased survival of HY4000 and UVS78 by <u>denV</u>. Strains as in Fig. 12. Note that the abscissa is expanded in the right-hand graph.

DISCUSSION

The denV gene was introduced to D. radiodurans by inserting the protein coding sequence into pS11, a duplication insertion vector that undergoes amplification under kanamycin selection due to the presence of the aphA gene (Smith et al., 1988). The denV gene was amplified as a non-selected passenger within the amplification unit. Although no known D. radiodurans promoter was adjacent, PD-glycosylase activity was detected within cell free extracts of transformants (Table 2).

The proper control for strains containing amplified chromosomal insertions of pPG100 (pS11 with denV) are those strains containing amplified pS11 (Table 1). In all instances, strains containing amplified insertions of pS11 did not express PD-glycosylase activity (Table 2) and had the same degree of UV-resistance as the parental strains lacking this amplified sequence (Figs. 12 and 13). Consequently, the increased UV survival of strains HY4002 (mtcA$^+$uvsE$^-$aphA$^+$denV$^+$) and HY3002 (mtcA$^-$uvsE$^-$aphA$^+$denV$^+$) can not be attributed to the presence of the chromosomal amplification per se, but instead the presence of denV itself.

HY3002 (mtcA$^-$uvsE$^-$aphA$^+$denV$^+$) demonstrated a decreased slope of the exponential portion of the survival curve by about 4-fold, as compared to UVS78 (mtcA$^-$uvsE$^-$) or HY3001 (mtcA$^-$uvsE$^-$aphA$^+$). To reduce survival to 10^{-2}, HY3002 required a 5-fold greater UV-fluence than UVS78 and HY3001 (Fig. 13).

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Wildtype, however, had much greater survival than HY3002, with a large shoulder extending to about 700 J/m^2, and a slight decrease in slope of the exponential portion of the survival curve as compared to HY3002. The fluence required to reduce wild-type survival to 10^{-2} was 4-fold greater than that required for HY3002 (Fig. 13). Quantitation of the extent of repair from these survival data is speculative; one possible interpretation, based on total fluences required to achieve survival of 10^{-2}:

$$\underset{5\text{-fold}}{\underset{(\underline{mtcA}^-\underline{uvsE}^-)}{\overset{UVS78}{}}} < \underset{(\underline{mtcA}^-\underline{uvsE}^-\underline{aphA}^+\underline{denV}^+)}{\overset{HY3002}{}} < \underset{4\text{-fold}}{\underset{(\underline{mtcA}^{\pm}\underline{uvsE}^{\pm})}{\overset{HY2000}{}}}$$

is that the PD-glycosylase in HY3002 is enabling repair of about half of UV-induced DNA damage normally repaired by the UV endonuclease-α and UV endonuclease-β excision pathways. Comparison based solely on the slopes of the exponential portion of the survival curves indicates that PD-glycosylase enables repair of a larger fraction than this; however, such an evaluation ignores the remarkably large shoulder of wild-type.

The specificity of T4 PD-glycosylase has been extensively investigated and no substrate for its glycosylase activity other than cyclobutane dimers has been identified (Chenevert et al., 1986 and citations therein). About 10% of dipyrimidine photoproducts are 6-4'-[pyrimidin-2'-one]-pyrimidines (Patrick, 1977; Brash and Hazeltine, 1982). While PD-glycosylase is not known to recognize this lesion, it is removed by excision

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repair initiated by the UvrABC protein of E. coli (Franklin and Hazeltine, 1984). This UV photoproduct, and others (Duker and Gallegher, 1988) that are presumably recognized by UV endonuclease-α and/or UV endonuclease-β may partially account for the discrepancy in UV survival between HY3002(mtcA⁻uvsE⁻ aphA⁺denV ⁺) and D. radiodurans strains wild-type with respect to excision repair. Another contribution to this discrepancy may be a low level of expression of the amplified denV gene in D. radiodurans, since there is no known proximal upstream promotor and this gene is probably transcribed by readthrough from a transcriptional unit in the D. radiodurans portion of pPG100 which may be distant from the denV insert. A third alternative is that D. radiodurans gene product(s) required for repair following incision by PD-glycosylase (but not the endogeneous UV endonucleases) is/are rate limiting.

Strain HY4000 (mtcA⁺uvsE ⁻), which contains only UV endonuclease-α, is significantly less resistant to UV than wild-type, and this discrepancy is almost eliminated by the presence of PD-glycosylase (Fig. 13). This observation is consistent with findings of Moseley and Evans (1983) that in mtcA⁺uvsC ⁻, mtcA⁺uvsD ⁻, and mtcA⁺uvsE ⁻ strains, dimer excision is 4 to 7 times slower than in wild type cells. One explanation for both the current observations and those of Moseley and Evans is that UV endonuclease-α has a relatively low affinity for one or more types of cyclobutane dimer in DNA. UV endonuclease-α has broad substrate specificity, initiating

repair of a variety of chemical lesions as well as UV damage (Tempest and Moseley, 1980; Moseley and Evans, 1983). In contrast, to date UV endonuclease-β is known only to contribute to repair of UV-induced damage. In the absence of UV endonuclease-α, UV endonuclease-β (in mtcA⁻uvsCDE⁺ strains) is sufficient to produce resistance to UV that is wild-type and excision of dimers from chromosomal DNA as rapid as wild-type (Moseley and Evans, 1983). Evolutionary retention of the uvsCDE genes encoding UV endonuclease-β for the exigency of UV damage suggests that UV endonuclease-α is qualitatively different with respect to substrate affinity and possibly mode of incision.

Is UV endonuclease-α, with its broad substrate range functionally similar to the UvrABC protein of E. coli, and UV endonuclease-β, with its substrate range apparently restricted to UV damage, a pyrimidine dimer-DNA glycosylase? While UV endonuclease-α has not been detected in vitro, UV endonuclease-β has been partially purified, and shown to be a 36 kd protein that incises UV irradiated DNA, producing the same number of incisions as PD-glycosylase, suggesting that UV endonuclease-β recognizes and cleaves pyrimidine cyclobutane dimers (Evans and Moseley, 1985). Although the cleavage mechanism of UV endonuclease-β has not been determined, the partially purified protein has been reported to not be a PD-glycosylase, using the same photoreversal assay employed in the current work (Evans and Moseley, 1988). Although not quite wild-type in UV

survival, strains expressing only UV endonuclease-α are still extraordinarily UV resistant. Thus, it might not be the case that repair of UV-induced damage is the selective basis for retention of the uvsCDE genes. An alternative reason for conservation of these genes by D. radiodurans is that UV endonuclease-β recognizes a form of damage not yet identified that is accrued in the soil habitat of this organism.

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